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USE OF THE BENZIDINE STAINING METHOD FOR THE STUDY OF CAPILLARIES IN THE CORNEA[1]

BY JOHN A. ZIEGLER[2]

Abstract

A review of the use of the benzidine stain is presented and a technique for making visible the capillary vessels in the excised animal cornea is described. Several hundred corneas of many different species of animals have been satisfactorily stained by this method. The method permits differentiation between filled and unfilled vessels and provides a means for demonstrating vascular permeability.

Introduction

The early investigations to make visible the normal vascular pattern of tissues were carried out by the method of injection (18, 19, 20). The application of injection methods, however, is limited because of the inherent disadvantages: (*a*) the uncertainty of securing adequate injection because of the arbitrary nature of the pressure employed; and (*b*) the inevitably unnatural picture obtained because the tissue injected does not show the true calibre of the vessels and may frequently display artificial extravasations.

Lepehne (13) was the first to attempt to stain selectively the haemoglobin in the vessels in tissue sections by applying the benzidine test for haemoglobin. Several recent discussions on the efficacy of this test appear in the literature (6, 9, 10, 11, 12, 15, 24). Lepehne observed that the resultant picture resembled that obtained by the injection method. Somewhat later, Sjöstrand (23) employed the benzidine stain in studying the blood depots in lungs and liver, and Lindgren (14) used it in investigating the number of capillaries in the brain tissue of young and of old persons. Pickworth (21), however, by using thick frozen sections, was the first to adapt the principle of the method specifically for demonstrating the cerebral vascular pattern. The technique, known as the "Pickworth–Lepehne method" was used in somewhat abbreviated fashion by Campbell, Alexander, and Putnam (2) and by Alexander and Campbell (1) in their studies of brain lesions. Kastein and Haex (8) made use of the benzidine stain in the histopathology of Weil's disease, while Kastein (7) studied the benzidine staining of blood vessels, and Sahs and

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[2] *Graduate Student.*

Alexander (22) described the vascular pattern of certain intracranial neoplasms by means of the benzidine stain. Fazio and Sacchi (5), and Fazio (4) recently studied the limits of applicability of the method in the investigation of circulatory phenomena of the central nervous system. Pfaff and Williams (17) have reported on the use of the benzidine stain for thick sections.

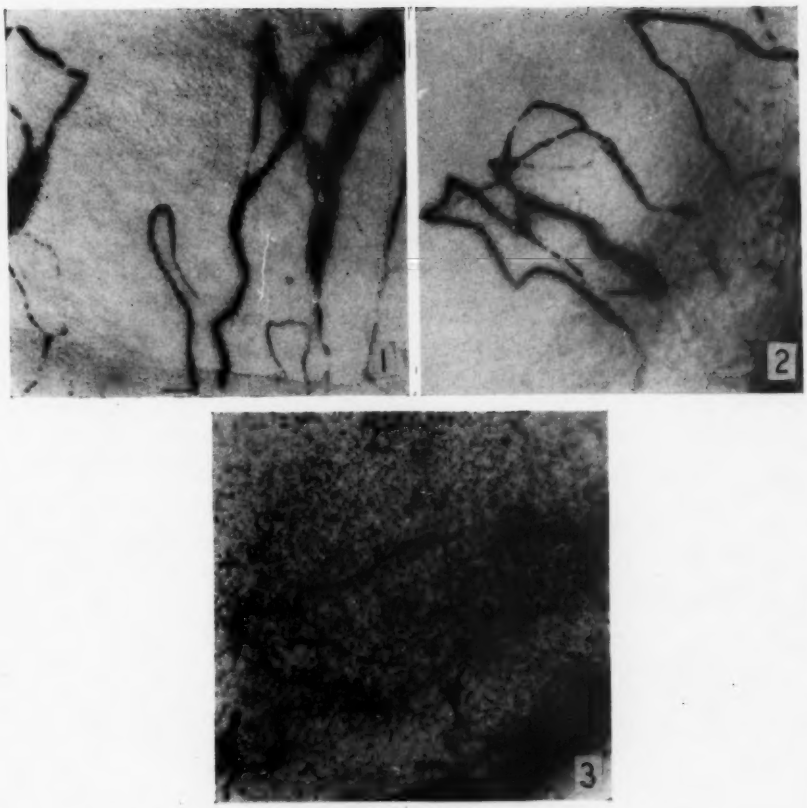
During a research on the metabolism of riboflavin, to be reported later, it became necessary to study the capillaries of several hundred rat corneas. Conventional staining techniques failed to permit adequate differentiation of the vessels from the remainder of the corneal tissue. The injection method using heparinized india ink was tried, but for reasons also to be reported later, it presented a wholly artificial and erroneous picture. Finally, an adaptation of the benzidine staining technique of Doherty, Suh, and Alexander (3) was employed and has given satisfactory results in nearly one thousand corneas including those of different animal species.

Methods

The technique will be described for a rat cornea but is essentially identical for the cornea of any species. The rat was anaesthetized by intraperitoneal injection of nembutal and the eye surgically removed. The whole eye was placed in 10% formalin–saline fixative and transferred to fresh fixative after two hours, where it remained *not less* than one week. When fixation was complete the eye was washed briefly and the cornea excised by careful dissection along the scleral margin of the limbus. Four equally spaced centripetal incisions in the cornea enabled it to be flattened out to a maltese-cross–shaped tissue. The cornea was washed for one-half hour in frequently changed distilled water and was stained in Solution *A** for 20 min. after which it was washed for 10 to 15 sec. in distilled water. The cornea was transferred to Solution *B** at room temperature for 15 min., and then placed in an incubator at 37° C. during which time the blue-green background faded to faint yellow while the blood vessels became jet black. The period of incubation was usually 30 min. but was never prolonged beyond 45 min. After thorough washing in distilled water (about 15 min.) the cornea was dehydrated in 70 and 95% alcohol each acidified with 2% acetic acid, followed by a final dehydration in neutral absolute alcohol. Clearing and mounting were performed simultaneously by use of the mounting medium described by McClung (16, p. 40).

* *Solution A*
Mix and dilute to 100 ml.:
0.5 gm. recrystallized benzidine in 50 ml. absolute alcohol.
0.1 gm. sodium nitroprusside in 10 ml. distilled water.
Prepare fresh each time.

Solution B
Dilute to 100 ml.:
50 ml. absolute alcohol.
3 ml. glacial acetic acid.
0.5 ml. 30% hydrogen peroxide.
0.1 gm. sodium nitroprusside.



FIGS. 1 and 2. *Vascularization and vascular permeability in the rat cornea.*
FIG. 3. *Vascularization in the rat cornea.*

Results and Discussion

Several typical results are presented in the accompanying photographs (Figs. 1 to 3) illustrating capillary invasion of the rat cornea. The stained corneas must be protected from undue exposure to direct sunlight, otherwise they will become bleached. An attempt to stain an incompletely fixed cornea generally results in an over-all deeply blue-stained specimen in which the vessels may be seen, but which is not suited to microphotography. While the duration of fixing is not critical, best results are obtained with a one to two week period, but corneas have been satisfactorily stained after periods of as much as seven months in formalin–saline. Occasionally a cornea retains a brownish background after staining and although the vessels appear dark brown the explanation for this is not apparent. It is essential that freshly prepared solutions be used each time, and that the benzidine be purified by recrystallization from alcohol.

It will be noted that the vascular pattern made visible by this technique resembles that obtained by the injection method but is without the disadvantages of the latter. Apparently only haemoglobin-containing cells and polymorphonuclear leucocytes are stained by benzidine, so that it is thus possible to differentiate between filled and unfilled but patent blood vessels in the cornea. The filled vessels present a jet black pattern whereas the unfilled vessels appear with only a faint brownish outline. Moreover, vascular permeability may be admirably demonstrated by this technique since the extravasation of haemoglobin or of leucocytes (or both) produces a diffuse area at the site of diapedesis.

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THE PROTHROMBIN LEVELS OF ANIMALS IN SHOCK[1]

By Ursula Irish[2], Jean I. Hamilton[3], R. E. Haist[4], and L. B. Jaques[5]

Abstract

Shock was produced in rats by a clamping technique and in dogs by the application of pressure cuffs to the hind limbs. Blood samples obtained from the shocked animals tended to clot in the presence of normally adequate amounts of heparin or oxalate. The prothrombin time, plasma time, clotting time, and prothrombin titre of the blood were determined before and during shock. The chief change noted in the clotting system was a more rapid activation of prothrombin in the samples from the shocked animals and from clamped controls. The cause of this change has not been established. It does not seem to be related to the activity of a proteolytic enzyme.

Studies of the changes occurring in animals in which shock was produced by compression of the limbs have been reported previously from this laboratory. It was observed that during the development of shock, samples of blood taken from these animals frequently clotted on standing, despite the fact that normally adequate quantities of oxalate or heparin had been added. For this reason, the clotting system in blood from the shocked animals was investigated.

Materials and Methods

Rats were shocked by a clamping technique (2) and dogs by the application of pneumatic pressure cuffs to the limbs (3). Blood samples were obtained by needle from the exposed heart in rats, and from the vein in dogs. The anticoagulant used was 0.1 cc. of 1.38% sodium oxalate solution per 0.9 cc. of blood. This was contained in the syringe into which the blood was drawn. In some experiments, for three days prior to the test each rat received by intraperitoneal injection 6 mgm. daily of vitamin K (2-methyl-1, 4-naphthoquinone) dissolved in corn oil. This dose is greatly in excess of the minimal daily requirement.

The prothrombin time (8) was determined at 37° C. with 0.1 cc. of the plasma to which was added 0.1 cc. of thromboplastin and 0.1 cc. of 1.11% calcium chloride solution. The thromboplastin was prepared by heating 0.5 gm. acetone-extracted rabbit brain in 10 cc. of saline to 56° C. for 15 min. and taking the supernatant suspension.

The prothrombin titre of the blood was determined by the two-stage technique as modified by Herbert (4). The oxalated plasma was diluted 1 : 25, 1 : 50, and 1 : 100 with physiological saline, and 1 cc. of each of these

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dilutions was incubated at 25° C. with 1 cc. M/4 imidazole buffer (pH 7.25) and 2 cc. of rabbit brain extract containing calcium. The amount of thrombin formed was assayed by taking 0.2 cc. of the incubation mixtures, adding to it 0.1 cc. of the fibrinogen solution and determining the clotting time at 37° C. The thromboplastin was prepared by the method of Herbert. Acetone-extracted rabbit brain (0.05 gm.) was made into a smooth emulsion with 5 cc. calcium–saline (0.05 gm. anhydrous calcium chloride and 0.855 gm. sodium chloride per 100 cc. water). This was centrifuged and the supernatant diluted with three times its volume of calcium–saline. Both the thromboplastin solutions were made up as required. We are indebted to Professor E. J. Cohn for a supply of lyophilized human fibrinogen; 100 mgm. of the dry material, containing 54% protein and 46% salts, was dissolved in 10 cc. of buffered oxalated saline and dialyzed against buffered oxalated saline, and then against buffered saline.

As shown previously (5), a linear relationship between clotting time and thrombin concentration is obtained when these are plotted on logarithmic paper. We are indebted to Dr. W. Seegers of Parke, Davis, and Co. for a supply of purified thrombin, containing 120 units (Iowa units) per milligram. A standard curve was constructed, using this standard thrombin preparation. This was used to express the prothrombin content of the oxalated plasma as equivalent thrombin units. The assigned value for the standard was checked both by the clotting time with the fibrinogen (one unit clotted the fibrinogen in 14.8 sec.) and by determining the prothrombin content of the blood of normal dogs (average was found to be 270 units/cc.). The highest thrombin value reached in a series of determinations is reported as the prothrombin titre. The rate of activation of the prothrombin was also followed directly, as described by Herbert, by observing the time at which fibrin appeared in the incubation mixture. This, according to Herbert, corresponds to the period of maximum activation.

To determine the clotting time, 2 cc. of blood was drawn into a syringe washed with saline. The first 0.3 cc. was discarded and then approximately 1 cc. of the blood was put into an 8 mm. tube and allowed to clot at 37° C. The tube was tipped at intervals to determine the end-point for clotting. For the plasma time, 0.2 cc. oxalated plasma was added to 0.2 cc. of a 1.11% solution of calcium chloride and the clotting recorded at 37° C.

Results

Clotting Time, Plasma Time, and Prothrombin Time in Dogs Before and After Shock

The results of the determinations in a series of animals are shown in Table I. There is a decrease in the clotting time of blood in five of seven dogs. This decrease may be due in part to the difficulty in obtaining samples of blood after shock has developed. This difficulty may increase the amount of thromboplastin in the sample and hence shorten the clotting time. The changes in the clotting times for recalcified plasma (plasma times) were rather

TABLE I

CLOTTING TIME, PLASMA TIME, AND PROTHROMBIN TIME IN DOGS SHOCKED BY THE APPLICATION OF PRESSURE CUFFS

Dog	Before application of cuffs	Before removal of cuffs	After removal of cuffs	Final sample after removal of cuffs	B.P.* at time of final sample, mm. Hg
Clotting time					
1	7 min.	6 min.	3 min.	1 min.	At death
2	3 " 25 sec.	3 " 35 sec.	3 " 32 sec.	2 "	70
3	3 " 15 "	3 " 30 "	—	3 " 30 sec.	At death
4	3 " 30 "	3 " 15 "	—	2 " 50 "	70
5	4 " 45 "	4 "	—	3 "	50
6	3 " 30 "	3 " 40 "	Died	—	
7	4 "	4 " 20 "	2 min. 40 sec.	3 min. 10 sec.	100
8**	4 " 15 "	4 " 30 "	—	4 " 20 "	50
Plasma time					
1	8 min.	8 min. 50 sec.	3 min. 20 sec.	2 min. 20 sec.	
2	Lost	3 " 50 "	3 " 30 "	3 "	
3	2 min. 20 sec.	2 " 25 "	—	2 " 10 "	
4	3 min. 30 "	1 " 20 "	—	1 " 35 "	
5	2 " 30 "	1 " 20 "	—	1 " 20 "	
6	45 "	45 "	Died	—	
7	1 " 20 " ***	3 "	2 min. 15 sec.	2 min. 25 sec.	
8**	13 " 30 "	3 " 30 "	—	4 " 20 "	
Prothrombin time					
1	22 sec.	19 sec.	18 sec.	18 sec.	
2	20 "	19 "	17 "	18 "	
3	25 "	24 "	—	30† "	
4	22 "	19† "	—	23† "	
5	20 "	18 "	—	23 "	
6	25 "	20 "	Died	—	
7	25 "	22 "	22 sec.	24 "	
8**	9 min. 50 "	7 min. 40 "	—	5 min. 25 "	
Average omitting ** and ***	23	20	19	23	

* *B.P. = blood pressure.*

** *Given 10 mgm./kgm. dicumarol 48 hr. previously.*

*** *Taken from damaged radial.*

† *Partly clotted before testing.*

variable, but some were shortened after removal of the cuffs. Here again, one factor involved in the differences may have been the difficulty with which the sample was obtained. The prothrombin times (Quick) were shortened too, but it is to be noted that this shortening occurred during the period of application of the cuffs when sampling difficulties were not encountered.

After release of the cuffs no further consistent decrease was evident. Difficulty was encountered in determining the prothrombin time in three samples from shocked animals because, on standing, the plasmas became partly clotted before the determinations were made. This was in agreement with the previous observation that the quantity of oxalate normally used was not sufficient to prevent clotting in some of the blood samples obtained from the animals in shock. One animal was treated with dicumarol 48 hr. prior to the experiment. In this animal the shortening of the plasma time and prothrombin time was very definite, although here, too, the shortening in plasma time occurred before the cuffs were removed.

Prothrombin Determinations in Rats Before and After Shock

While in the dogs the Quick prothrombin times were fairly consistent, in the rat experiments the results (cf. Table II) were much more variable even in control animals. In a second series of rats, synthetic vitamin K was

TABLE II

PROTHROMBIN DETERMINATIONS IN NORMAL AND SHOCKED RATS

Quick prothrombin time, sec.				Herbert prothrombin titre, units		
Control	Shock	Control*	Shock*	Control*	Clamped control*	Shock*
20	85	75	47	350	340	176
65	72	85	45	360	340	275
32	57	43	28	285	340	230
48	55	86	—	340	340	295
85	55	90	71	370	380	340
69	—	99	—	380	400	315
35	—			340	355	345
58	39			285	330	—
73	60					
65	55					
52	55					
70	50					
56	58	80	48	339	353	282

* *Vitamin K (6 mgm.) administered daily for three days before the experiment.*

administered to both the test and control groups. No differences between the average prothrombin times of the animals in shock and of control animals were found. Too few animals were used to warrant attaching any significance to the higher average value for the control rats treated with vitamin K.

In view of the great variability of the prothrombin time in rats, as measured by the Quick procedure, in further experiments the prothrombin titre was determined by the two-stage technique. The results are reported in Table II. The average prothrombin value was slightly lower for the shocked rats than for the controls, though the range was wide. There was no marked difference between the average values for the two control groups.

In preliminary experiments it was observed that activation of the prothrombin in the plasma sample taken after shock always occurred earlier than in the control plasma set up at the same time. The time of appearance of the fibrin thread in these samples was therefore determined accurately (Table III). As can be seen from the table, there is a marked difference between the shocked and control (non-clamped) animals. The dilutions of the 'shock' plasma activated much more rapidly than the corresponding dilutions of the control plasma. However, control animals from which the clamps were not removed, i.e. 'clamped controls', showed an increase in the rate of activation similar to that observed in the shocked animals.

Discussion

As stated above, a change in the clotting system of the blood after shock was first indicated by the fact that oxalate and heparin did not always prevent the blood from clotting on standing in the cold. This finding has been confirmed in the present experiments. It was observed also that in blood from shocked animals there was a decrease in the clotting time, plasma time, and prothrombin time, providing further indication of increased coagulability. However, it was noted that the decrease in prothrombin time occurred before the cuffs were removed, i.e. before shock developed. The decrease in clotting time and plasma time occurred after removal of the cuffs but seemed to result, in part at least, from the greater difficulty in obtaining the blood sample. Mylon *et al.* (6) report a marked shortening of the clotting time immediately after removal of the cuffs in some animals. The effect was marked in 30 sec. and the clotting time had returned to normal in eight minutes. Associated with this change was a sudden sharp fall in blood pressure followed by a temporary rise. The changes in clotting studied here are those associated with the later slow fall in blood pressure.

The shortened prothrombin time (Quick) might be considered to indicate an increase in prothrombin concentration in the blood. Actual determinations of concentration (by the two-stage technique) indicate that there is a slight decrease in prothrombin concentration after shock. An increase in the rate of activation of prothrombin occurs, however. This change too is observed before removal of the clamps or before shock develops. Hence it is not necessarily associated with the shock syndrome.

Quastel and Racker (7) report that extracts of a rat's limb to which a tourniquet had been applied had a greater thromboplastic activity than extracts from the opposite normal limb. This harmonizes with the finding that the shocked rats in our experiments showed an increased rate of activation of prothrombin. However, since a similar change was observed in clamped control animals (unshocked) it seems unlikely that some material from the anoxic tissue is causing this change in the blood. There is the possibility, of course, that, in the clamped animals, a small strip of tissue at the proximal edge of the clamp will have its circulation reduced and may contribute to the phenomenon.

TABLE III

'ACTIVATION TIME' OF DILUTED PLASMA FROM CONTROL AND SHOCKED RATS

Controls			Clamped controls			Shocked		
Dilution								
1 : 25	1 : 50	1 : 100	1 : 25	1 : 50	1 : 100	1 : 25	1 : 50	1 : 100
Activation time								
2 min. 25 sec.	4 min. 36 sec.	*	2 min. 0 sec.	3 min. 40 sec.	6 min. 0 sec.	2 min. 55 sec.	6 min. 25 sec.	*
2 " 52 "	5 " 42 "	*	2 " 2 "	3 " 25 "	7 " 23 "	2 " 15 "	5 " 0 "	8 min. 30 sec.
2 " 58 "	6 " 5 "	*	1 " 45 "	4 " 0 "	7 " 25 "	1 " 50 "	3 " 20 "	5 " 0 "
3 " 35 "	8 " 0 "	*	3 " 0 "	4 " 50 "	7 " 0 "	3 " 0 "	4 " 45 "	7 " 10 "
2 " 45 "	5 " 20 "	*	2 " 15 "	3 " 35 "	7 " 20 "	2 " 35 "	5 " 0 "	8 " 10 "
4 " 30 "	*	*	2 " 30 "	4 " 0 "	7 " 0 "	2 " 53 "	4 " 0 "	6 " 40 "
4 " 20 "	8 min. 0 sec.	—	2 " 50 "	4 " 0 "	7 " 40 "	2 " 55 "	4 " 50 "	7 " 45 "
			2 " 50 "	4 " 45 "	7 " 10 "			
3 min. 21 sec.	6 min. 17 sec.	*	2 min. 24 sec.	4 min. 2 sec.	7 min. 7 sec.	2 min. 38 sec.	4 min. 46 sec.	7 min. 12 sec.

* *Activation time was 10 min. or more and therefore the end point was too poor to judge activation time accurately.*

The shortened Quick prothrombin time, the shortened plasma and clotting times in a number of dogs, and the consistently more rapid activation of prothrombin in the rats suggest the presence in the blood of the animals of a powerful thromboplastin. It is of interest that in the dog treated with dicumarol the plasma time became normal in shock and hence much shorter than the prothrombin time. Recent studies by Ferguson and others have emphasized the importance in clotting of the thromboplastic factors in plasma. It appears that some change in the activity of these factors occurs in the blood of clamped dogs and rats. However, no "thromboplastic enzyme" activity, either in dogs or rats, could be detected in the samples of oxalated plasma removed for prothrombin determinations before and after shock when measured by the power to destroy fibrinogen and lyse fibrin (1). Activation of the dog plasma with chloroform (9) gave a powerful proteolytic enzyme as measured by the same test. The same treatment of the rat plasma gave no measurable proteolytic enzyme. A marked difference between dog and rat plasma is seen, therefore, since chloroform caused the rat plasma to clot in about two hours and lyse in 30 hr., while dog plasma showed a trace of clot in three minutes and lysed in 10 min. No significant difference in the enzyme activity of the chloroform plasma could be detected in the dog before and after clamping. Lysis of the chloroform plasma from the rat tended to occur earlier in the normal control samples than in the samples obtained from the shocked animals rather than the reverse. Hence the increased thromboplastic activity demonstrated in the increased rate of activation does not appear to be related to the proteolytic enzyme.

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AN IMPROVED PROCEDURE FOR THE BIO-ASSAY OF PITUITARY EXTRACT (POSTERIOR LOBE)[1]

By W. M. Bachinski[2], M. G. Allmark[3], and C. A. Morrell[4]

Abstract

A more efficient design, based on a graded response technique, for the bio-assay of pituitary extract (posterior lobe) is presented. Eight longitudinal strips of uterine muscle from one virgin guinea pig are employed. Two dosage levels are used, differing by a fixed percentage, for both the standard and the unknown preparations. Doses are added to the bath in a random order. Factorial analysis of the responses gives the most probable potency of the unknown preparation, and the standard error of this observed potency is found by means of the analysis of variance. The method has the advantages of speed and accuracy. As many as four unknown extracts have been assayed in one day. For 23 unknown solutions the mean actual error was 7.7%, and the mean standard error was 3.8%.

The present official method in Canada and the United States for the biological assay of pituitary extract (posterior lobe) is wasteful in time and guinea pigs and does not provide a means of estimating the reliability of individual assays. A method based on a quantal type of response was previously published by this laboratory (1). Although possessing several advantages over the official method, it lacks the precision obtainable with a graded type of response. The proposed method outlined in this paper employs a graded response technique (2). It has the advantages of speed and accuracy. All guinea pigs gave satisfactory results and as many as four unknown extracts have been assayed in one day.

In conducting an assay the procedure is as follows. Eight longitudinal strips of uterine muscle are obtained from one guinea pig. The method of preparation of the strips and the salt solution used has been previously described (1). The apparatus has been modified by the inclusion of a more efficient preheater system and a tissue chamber with the inlet near the base.

A dilution of the standard preparation is added to the bath to produce a contraction of all the strips. Alternate doses of the standard and unknown preparations are used to determine the approximate potency of the unknown. On the basis of this approximate potency the low (U_1) and high (U_2) doses of the unknown are adjusted to give almost the same response as the doses of the standard extract (S_1, S_2). When orienting, it is important that the total sum of responses to the standard preparation be submaximal.

In practice a sum total of responses from 30 to 60 mm. for the eight strips gives a good margin of safety. A logarithmic interval of 0.3 or 0.2 between

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the low and high doses is used for the two dose design. Dilutions of the standard extract 1 : 10 to 1 : 200 may be used for assay purposes, but a dilution of 1 : 50 has been found satisfactory in most cases. Experiments with various time intervals have shown that an eight minute period between the addition of doses gives satisfactory results. This time interval allows four minutes for responses, one and one-half minutes for change of solution, and a two and one-half minute rest period.

Following the adjustment of the low and high doses of the standard and unknown, doses are added in a random order. Each dose is administered to the eight strips in the bath until all four doses are given, completing one group of test doses. To increase the precision of the assay three or more groups of test doses may be given, although a fair degree of accuracy has been obtained with one group of test doses.

In the example shown in Table I the strength of one unknown preparation was assayed against the Canadian Standard Pituitary Extract. The figures in the first five lines in the table were used in orienting the doses. In Table V are given the results of other assays conducted on solutions whose potency was unknown to the operator.

TABLE I

DETAILS FROM A TYPICAL ASSAY

Tracing 16. Virgin guinea pig. Weight 360 gm., in oestrus state

Dose No.	Preparation	Dose, cc.	Strip number								
			1	2	3	4	5	6	7	8	
			Response, mm.								
1	*S*/200	0.35	1	2	4	0	7	17	15	4	
2	*U*/200	0.35	0	1	0	0	3	1	0	0	
3	*S*	0.35	1	3	7	2	5	11	17	13	
4	*U*	1.65	1	3	6	2	5	10	10	8	
5	*U*	1.65	1	3	8	2	6	8	5	11	
6	S_1	0.35	3	3	5	2	4	2	16	10	
7	U_2	3.72	7	8	21	20	7	17	25	18	
8	U_1	1.86	1	3	1	1	4	4	1	7	
9	S_2	0.70	6	8	19	16	7	12	22	13	293
10	U_1	1.86	0	4	0	4	5	5	2	7	
11	S_2	0.70	8	8	19	16	5	18	26	14	
12	S_1	0.35	0	3	0	4	4	4	2	5	
13	U_2	3.72	11	9	9	13	7	18	27	13	270
14	S_1	0.35	0	1	0	2	3	0	1	0	
15	U_2	3.72	3	6	21	16	5	17	24	20	
16	U_1	1.86	1	2	0	3	3	1	1	0	
17	S_2	0.70	3	6	7	15	5	18	8	17	209
18	*S*	20.00	18	26	25	25	19	22	31	30	

The data in lines 6 to 17 in Table I are rearranged in Table II for calculation.

TABLE II

REARRANGED DATA

Muscle strip	S_1	S_2	U_1	U_2	Totals	
1	3	6	1	7	17	
	0	8	0	11	19	
	0	3	1	3	7	43
2	3	8	3	8	22	
	3	8	4	9	24	
	1	6	2	6	15	61
3	5	19	1	21	46	
	0	19	0	9	28	
	0	7	0	21	28	102
4	2	16	1	20	39	
	4	16	4	13	37	
	2	15	3	16	36	112
5	4	7	4	7	22	
	4	5	5	7	21	
	3	5	3	5	16	59
6	2	12	4	17	35	
	4	18	5	18	45	
	0	18	1	17	36	116
7	16	22	1	25	64	
	2	26	2	27	57	
	1	8	1	24	34	155
8	10	13	7	18	48	
	5	14	7	13	39	
	0	17	0	20	37	124
Totals	74	296	60	342	772	

The total variation in the experiment is first broken down into its components by the analysis of variance. A simple outline of the procedure is as follows:

$$\text{Correction term} = \frac{(772)^2}{96} = 6208.17$$

$$\text{Total sum of squares for muscle strips} = 11324 - 6208.17 = 5115.83$$

$$\text{Sum of squares for differences in muscle strips} = \frac{(43)^2 + (61)^2 + \ldots (155)^2 + (124)^2}{12} - 6208.17 = 863.16$$

$$\text{Sum of squares for differences in doses} = \frac{(74)^2 + (296)^2 + (60)^2 + (342)^2}{24} - 6208.17 = 2694.16$$

$$\text{Sum of squares for changes in group sensitivity} = \frac{(293)^2 + (270)^2 + (209)^2}{32} - 6208.17 = 117.77$$

TABLE III

ANALYSIS OF VARIANCE

Source of variation	Degrees of freedom	Sum of squares	Variance	F
Total	95	5115.83	53.85	
Doses	3	2694.16	898.05	51.73
Muscle strips	7	863.16	123.31	7.10
Group sensitivity	2	117.77	58.89	3.39
Remainder	83	1440.74	17.36	

$S = \sqrt{17.36} = 4.166$

TABLE IV

FACTORIAL ANALYSIS OF DATA FROM TABLE II

Source of variation	Factorial coefficient (x) for dose				Divisor $NS(x^2)$	Sum of products $S(xY\rho)$	Variance $\frac{S^2(xY\rho)}{NS(x^2)}$
	S_1	S_2	U_1	U_2			
Difference between samples	−1	−1	+1	+1	96	+ 32	$10.67 = D^2$
Slope of curve	−1	+1	−1	+1	96	+504	$2646.00 = B^2$
Lack of parallelism	+1	−1	−1	+1	96	+ 60	37.50
Total number of responses	74	296	60	342			

$D = \sqrt{10.67} = 3.267$ $\qquad$ $B = \sqrt{2646.0} = 51.44$

The significance of the variance ratios in Tables III and IV may be obtained from the tables of Fisher and Yates (3). The variance ratios from Table IV are as follows:

For difference between samples $\frac{10.67}{17.36} = 0.62$

For slope $\frac{2646}{17.36} = 152.42$

For parallelism $\frac{37.50}{17.36} = 2.16$

The potency is computed as follows:

$M = \bar{x}_s - \bar{x}_u + \frac{kID}{B}$, where $k = 1$ and $I =$ the logarithmic interval between doses

$M = 0.54407 - 1.26951 + \frac{(0.3010)\ (3.267)}{51.44} = -0.7063$ or $\bar{1}.2937$

The potency in per cent $= 100$ (antilog M) $= 100\ (0.197) = 19.7$

The standard error of M is calculated as

$$S_m = \frac{skI\sqrt{B^2 + D^2}}{B^2}$$

$$S_m = \frac{(4.166)\ (0.3010)\sqrt{2646.00 + 10.67}}{2646} = 0.0244$$

The average standard error of the relative potency in per cent is calculated by means of Cochran's formula;

$$s.\ e. = 2.303 S_m\ (\text{antilog } M)\ 100$$

$$s.\ e. = (2.303)\ (0.0244)\ (0.197)\ (100) = 1.1\%$$

The estimated potency of the unknown is therefore $19.7\% \pm 1.1\%$ of the standard pituitary extract. The true potency was 20.0%.

TABLE V

ASSAYS OF SOLUTIONS OF KNOWN POTENCY

Assay No.	True potency, %	Potency found, %	Standard error, %	Actual error as % of true potency
1	57.0	56.7	3.6	0.6
2	57.0	52.8	3.4	7.4
3	95.0	91.2	4.5	4.0
4	95.0	91.3	4.0	3.9
5	80.0	85.5	4.4	6.9
6	80.0	73.4	4.1	8.3
7	35.0	32.9	1.7	6.1
8	35.0	34.6	1.7	1.1
9	46.7	54.4	2.3	16.5
10	46.7	42.3	3.7	9.4
11	46.7	39.9	4.3	14.6
12	100.0	105.7	7.6	5.7
13	66.7	76.8	4.3	15.1
14	66.7	63.8	2.7	4.3
15	66.7	80.7	4.6	21.0
16	20.0	19.7	1.1	1.5
17	20.0	22.5	1.2	12.5
18	20.0	20.7	0.8	3.5
19	80.0	75.4	4.2	5.8
20	80.0	82.1	2.6	2.6
21	133.3	152.0	5.6	14.0
22	133.3	146.5	9.0	9.9
23	133.3	137.8	6.5	3.4
			Mean 3.82	7.74

At the present time a further analysis of the data is being made to see whether other sources of variation are significant.

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THE EFFECT OF HEATING, THE PRESENCE OF ANTIOXIDANTS, AND THE LEVEL AND MELTING POINT OF THE FAT COMPONENT ON THE NUTRITIONAL VALUE OF DIETS AS INDICATED IN RAT FEEDING TESTS[1]

By E. W. Crampton[2] and M. F. Mills[3]

Abstract

Male white rats were fed baked and unbaked diets containing blended cottonseed oil of two different melting points (45° and 57° C.) with and without antioxidant. The fat was incorporated in the diets at 4 and 16% levels. The relative nutritive value of the diets was measured by growth of rats, digestibility of the diet, and the proportion of fat deposited in the livers and carcasses.

The digestibility of the fat decreased as the melting point increased from 45° C. to 57° C. The addition of 0.1% nordihydroguairetic acid to the fat showed no effect on the nutritive value of the diets. Increasing the fat content of the diet from 4 to 16% resulted in a decline in body weight that can be accounted for only on the assumption of poor utilization of fat. Heating the diet lowers its efficiency for rats.

Introduction

Digestible fats contribute some two and a quarter times as much metabolizable energy to a diet as either carbohydrate or protein. Thus foods of high fat content are "richer" than low fat foods per unit of weight. Fatty meals, too, are absorbed more slowly than meals of low fat content, and thus have more staying power. Under some conditions a concentrated diet is desired for high energy value, but because of the properties of fats there is a limit to the extent to which they may be incorporated in the diet.

Their susceptibility to rancidity is one of the factors often limiting the usefulness of foods high in fat. Irwin, Weber, Steenbock, and Godfrey (5) report that the rate of absorption of a hydrogenated fat varied inversely with the degree of oxidation.

Many common shortenings are stabilized against rancidity with antioxidants. A study of antioxidant effectiveness of several compounds on vegetable fats and oils was made by Mattil, Filer, and Longenecker (6). The most effective single compound they found was gallic acid, although nordihydroguairetic acid (N.D.G.A.), ascorbic acid, and ascorbyl palmitate each about doubled the keeping time of any given vegetable fat or oil.

There is some dispute as to whether low melting point fats are nutritionally better than high melting point fats. It is popularly believed that when the melting point is high, digestion is lower, and there is a tendency for unabsorbed fats to appear in the feces. Steenbock, Irwin, and Weber (12) find that fats with a melting point above 37.8° C. are slowly absorbed.

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Hoagland and Snider (4) find no difference in digestibility coefficients of fats whose melting points are 56° C. and 39° C., and therefore report no consistent relation between the percentage of saturated fat acids in shortening or its melting point and the digestibility coefficient. Burr and Barnes (1) also find no correlation between melting point or fatty acid composition and digestibility.

Several workers report on the effect of heat on certain fractions of the diet. Morgan (8) finds a considerable decrease in biological value of whole wheat protein and wheat gluten after toasting. Cover, McLaren, and Pearson (2), in studies on the retention of the B-vitamins in 'rare' and 'well done' beef, find that the retention of thiamin and pantothenic acid is significantly lower in the latter case.

Specifically, the principal objectives of this research were: (*a*) to obtain data on the effect of the melting point of the fat and of the level of fat intake on the nutritional value of the diet; and (*b*) to study the effect of heat and the addition of antioxidants on the nutritional value of the diet.

Growth of rats and carcass examination, and digestibility of diets and diet components were used as criteria for judging the nutritional value of the diets.

Experimental

The general plan of this test was to feed young growing rats baked and unbaked diets in which were incorporated, at each of two different levels, fats of two different melting points (45° C. and 57° C.), with and without antioxidants. The relative nutritive value of the diets as fed was measured by the growth of the rats during the test period.

The feeding involved two replicate tests, each with 48 male white rats 25 to 30 days old. In each replicate the animals were allotted at random to individual wire bottomed cages grouped into 16 lots of 3 animals each. The test diet and water were provided *ad libitum*, but records of individual feed intake were taken. Supplements of vitamins A and D were administered twice weekly.

Inasmuch as the interest in this work lay in its applicability to the nutrition of humans, the ration fed consisted of ingredients that could be made into acceptable biscuits. The percentage composition of the high and low fat diets is given in Table I.

The two fats were prepared by blending cottonseed oil with cottonseed stearin in proportions to give melting points of 45° C. and 57° C. The low melting fat (iodine No. 95) contained 5% cottonseed stearin and 95% cottonseed oil. The high melting fat (iodine No. 43) consisted of 66.6% cottonseed stearin and 33.3% cottonseed oil. The antioxidant used was nordihydroguairetic acid and was incorporated into the fat at a level of 0.1%.

To prepare the heated diets, the ingredients were mixed with a minimum of water to make a biscuit dough and baked at 425° F. for 15 min. Subsequently the biscuits were oven-dried just below 100° C. and ground for feeding.

TABLE I

DESCRIPTION OF MIXTURES USED

Ingredient or fraction	Low fat	High fat
Formula		
Bread flour, %	81.5	70.0
Skim-milk powder, %	10.0	10.0
Fat, %	4.0	16.0
Salt, %	1.0	1.0
Baking powder, %	3.5	3.0
Analysis		
Protein, %	15.25	13.65
Fat, %	4.0	15.3
Ash, %	3.7	3.2
Moisture, %	9.8	8.0
Carbohydrate (by difference), %	67.2	59.8
Calculated metabolizable energy*, Cals./100 gm. feed	365.8	431.5

* *Using conventional values of 4, 9, 4 Cal./gm. of carbohydrate, fat, and protein, respectively.*

Individual records were kept of the gains in weight and feed consumption for the 28-day test period.

During the third week of the growth test, the feces from each rat were quantitatively collected for the determination of apparent digestibility coefficients. To obtain a measure of the metabolic fat needed in order to calculate the true digestibility of fat, two groups of five rats, comparable in size to those on the growth test, were subjected to a separate digestion trial in which a fat-free diet was used. A significant correlation between dry matter output and metabolic fecal fat was found. This relation was measured by a regression coefficient (b) of 33.6 gm. The metabolic fat for each rat of the digestion trial was then calculated according to the following equation:

$$M.F.F. = 27.1 + 33.6\,(x),$$

where $M.F.F.$ is metabolic fecal fat in milligrams and x is feces dry matter in grams.

At the conclusion of the growth trial the 48 rats were killed by stunning, and determinations of the crude fatty acid according to the method of Gavin and McHenry (3) were made on livers and on liver-free carcasses.

Results

(a) *Growth*

The mean 28-day gains according to feeding groups are presented in Table II.

A summary of the mean values for the initial weights of the rats at the start of the tests, their 28-day gains in live weight, and the 28-day feed consumption is presented in Appendix Table I. Statistical analysis of the

TABLE II

MEAN FEED INTAKE AND 28-DAY GAINS ACCORDING TO FEEDING GROUPS

Comparison	Group	28-day feed intake, gm.	28-day gains, gm.	
			As observed	Adjusted to average feed intake*
Fat levels	4%	222	28.4	24.6
	16%	177	13.8	17.5
Melting points of fats	45° C.	196	19.6	20.4
	57° C.	203	22.6	21.8
Stabilization of fats	Antioxidant	196	19.7	20.2
	Nil	204	22.4	22.0
Heat	Heated	173	11.4	15.3
	Unheated	227	30.8	26.9
Test average:		200	21.1	21.1

* *Differences between averages exceeding 3.6 gm. are statistically significant at a probability of 5%.*

individual data indicated a significant difference between the initial weights of the rats in the two tests. However, the design of the trial was such that the two replicates could be combined without bias in interpreting the data. Correlation studies indicated no significant relation between gains and initial weight. There was, however, a high correlation between gains and feed intake. Therefore, observed gains were adjusted by regression ($b = 0.1535$) to average feed intake in order to distinguish between differences in gains due to amounts of feed and those due to kinds of feed. Differences in these adjusted gains are presumably due to a difference in nutritive value of the feed. The growth results can be considered under the following headings.

Fat level.—The two levels of fat show marked differences in gains—the 16% being inferior to the 4% level. These results are rather difficult to interpret unless it is postulated that the fat used in this test was poorly utilized. Results (see later) indicate no difference in digestibility between the two fat levels, nor was a difference found in the percentage of crude fatty acids deposited in the body stores and livers. Therefore, it might be assumed that this fat did not contribute to body stores. Since diets containing fat have correspondingly less carbohydrate and protein, a failure to utilize fat would result in a decrease in energy value as the fat component was increased, and could account for the lower gains on the high fat diets. It is interesting to note in this connection, that Sinclair (11) found better growth on a fat free diet than on one in which the fat was elaidin.

Melting point.—The group averages show no marked difference in gains traceable to different melting points (45° C. and 57° C.). Apparently hard and soft fats were equally efficient (or inefficient) as far as gains were concerned.

Antioxidants.—The addition of antioxidants to the fat showed no effect on live weight gains.

Heat.—The effect of heat on the diet is quite obvious. A diet heated at a temperature of 425° F. for 15 min. resulted in a significant decrease in gains as compared to the unheated diet. Morgan and King (9) credit somewhat similar results to a reduction in the biological value of the diet protein by heat.

It must also be remembered that in our studies this particular diet was the entire source of the vitamin-B complex. Therefore, the decrease in gains might be partially due to a destruction of the already meagre amounts of the heat labile parts of the B-complex in the diet. Further light is thrown on the subject by the analysis of the crude fatty acids of the bodies and livers.

(*b*) *Carcass Studies*

The mean values for percentage of crude fatty acids in livers and carcasses according to feeding groups is presented in Table III.

TABLE III

MEAN VALUES FOR PERCENTAGE CRUDE FATTY ACIDS OF LIVERS AND CARCASSES OF RATS

Groups	Crude fatty acids, %		
	Livers	Carcasses	Livers + carcasses
4% fat level	2.98	3.71	3.67
16% fat level	2.48	3.76	3.69
Low m.p.	2.81	4.48	4.39
High m.p.	2.64	2.99	2.98
Antioxidant	2.84	3.78	3.73
Nil	2.61	3.69	3.64
Heated	3.16	3.48	3.45
Unheated	2.29	3.99	3.92
Necessary difference for significance	0.18	0.75	
Test averages	2.73	3.73	

Level of fat.—There was no demonstrable effect of different levels of fat in the diet on the proportion of fat deposited in the carcasses. The lower fat diet, however, resulted in a greater proportion of fat in the livers. This is not easy to understand especially since the actual amount of fat deposited in the livers on the two different diets was the same. This test offers no explanation of this result.

Melting point.—When the diets contain fat of high melting point a statistically significant smaller percentage of the carcass weight is fat than when the fat in the diet is of low melting point. On examination of the actual amounts of fat deposited when the two kinds of fat are used in the diet, the same relation holds true. This is to be expected since the relative digestibilities of the two fats (see later) differ in the same way.

Antioxidant.—The use of diets containing the antioxidant resulted in the deposition of slightly greater quantities of liver fat. Statistically the difference is significant but nutritionally it would seem that this result should be inter-

preted as evidence against any damaging effect of the antioxidant on the feeding value of the diet.

Heat.—A geater proportion and amount of fat accumulated in the livers of rats fed heated diets, and (although statistically not significant) a suggestion of lower body fat resulted from the use of the heated diets. Some change detrimental to the nutritive value of the diet seems to have taken place during the baking process. This might indicate the destruction of some dietary factor (such as pantothenic acid) that plays a role in the mechanism for the transportation of synthesized fat from the liver to body stores. Schaefer, McKibbin, and Elvehjem (10) report fatty livers as one of the symptoms of pantothenic acid deficiency in dogs, and Cover, McLaren, and Pearson (2) find a significant loss of pantothenic acid in 'well-done' beef as compared to 'rare' beef.

(*c*) *Digestibility Studies*

The mean values of the digestibility coefficients obtained in this study are:

Dry matter	94.9%
Carbohydrate	97.2
Crude protein	88.0
Ether extract	93.5

The true digestibility of ether extract was 96.1%. In all these data the only factor in the diet treatment that had a significant effect on digestibility was the melting point of the fat used. The digestibility of the fat of lower melting point was found to be higher (96.7% vs. 90.4% apparent: 99.0% vs. 93.2% true). This might be due to the higher percentage of stearin in the fat of high melting point than in the low melting point fat (66.6% vs. 5%).

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APPENDIX TABLE I

MEAN VALUES FOR INITIAL WEIGHT, 28-DAY GAINS, AND FEED CONSUMPTION (GM.)

Fat level	Pre-treatment of diet	Variable recorded	Low melting point		High melting point	
			Anti-oxidant	Nil	Anti-oxidant	Nil
4%	Heated	Initial weight	64	74	73	68
		Gain	6.0	23.2	15.2	23
		Feed consumption	156	205	186	211
	Unheated	Initial weight	69	72	70	70
		Gain	45.9	29.0	38.0	46.7
		Feed consumption	273	229	245	253
16%	Heated	Initial weight	83	75	71	70
		Gain	0.7	5.3	12.0	5.7
		Feed consumption	160	146	164	154
	Unheated	Initial weight	69	70	71	61
		Gain	17.5	29.2	22.7	17.3
		Feed consumption	177	226	205	187

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